Anal. Chem. **2007,** *79,* 6592−6598

Photopatterning Enzymes on Polymer Monoliths in Microfluidic Devices for Steady-State Kinetic Analysis and Spatially Separated Multi-Enzyme Reactions

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A method for photopatterning multiple enzymes on porous polymer monoliths within microfluidic devices has been developed and used to perform spatially separated multienzymatic reactions. To reduce nonspecific adsorption of enzymes on the monolith, its pore surface was modified by grafting poly(ethylene glycol), followed by surface photoactivation and enzyme immobilization in the presence of a nonionic surfactant. Characterization of bound horseradish peroxidase (HRP) was carried out using a reaction in which the steady-state profiles of the fluorescent reaction product could be measured in situ and then analyzed using a plug-flow bioreactor model to determine the observed maximum reaction rate and Michaelis constant. The Michaelis constant of 1.9 μmol/L agrees with previously published values. Mass-transfer limitations were evident at relatively low flow rates but were absent at higher flow rates. Sequential multienzymatic reactions were demonstrated using the patternwise assembly of two- and three-enzyme systems. Glucose oxidase (GOX) and HRP were patterned in separate regions of a single channel, and product formation was analyzed as a function of flow direction. Significant product formation occurred only in the GOX to HRP direction. A three-enzyme sequential reaction was performed using invertase, GOX, and HRP. All possible arrangements of the three enzymes were tested, but significant product formation was only observed when the enzymes were in the correct sequential order. Photopatterning enzymes on polymer monoliths provides a simple technique for preparing spatially localized multiple-enzyme microreactors capable of directional synthesis.

Enzymes have been used in microfluidic systems to enable a variety of biochemical applications including DNA[1−3] and protein analysis,[4−7] measurement of enzyme kinetics,[8−12] biochemical analyte sensing,[13−16] and, more recently, combinatorial biosynthesis.[17,18] The use of enzymes in microfluidic devices is motivated by the significant reduction in the amount of reactant and catalyst required compared to bench-scale protocols and is often performed with the goal of integration within a larger lab-on-a-chip device. The utility of enzymatic reactions in microscale devices has been espoused in several recent reviews.[19−23] The use of immobilized enzymes is of particular interest because immobilization facilitates reuse of enzymes and simplifies product separation,

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10.1021/ac070705k CCC: $37.00
Published on Web 07/21/2007

as well as improves enzymatic reaction rates.[24] A final advantage of immobilization arises from the ability to localize the enzyme in a microfluidic environment whereby the direction and rate of fluid flow imparts the ability to control the sequence and residence time of multienzymatic reactions. Such a configuration also allows sequential reactions to occur within well-defined spatial reaction zones. Therefore, to realize all of these benefits, we sought to develop a method for patterning multiple enzymes within microfluidic reactors.

The complexity of confining multiple enzymes to distinct locations within a microfluidic system has thus far limited the development of spatially separated multienzymatic reactors. The first demonstration of a spatially separated enzymatic reaction in a microfluidic format was performed by Cremer and co-workers, where two enzymes, glucose oxidase (GOX) and horseradish peroxidase (HRP), were covalently immobilized to the surface of two separate PDMS channels. The two channels were connected externally using plastic tubing in order to carry out a sequential enzymatic reaction;[25] a similar approach was later used by Ku et al.[18] Crooks and co-workers immobilized the same two enzymes to the surface of microbeads that were retained in separate sections of a single microfluidic channel by microfabricated weirs.[26] While this method is extremely flexible, it does not scale well for multiple enzymes because each additional enzyme requires the fabrication of an auxiliary channel and weir to pack and retain the microbeads in the channel.

An attractive and readily scalable alternative involves the use of photochemistry to pattern enzymes in microfluidic channels. Photochemical approaches allow enzymes to be patterned in fully fabricated microscale devices via a process similar to photolithography. This process has been previously demonstrated for covalently immobilizing proteins or enzymes to the surfaces of empty channels.[27−29] Because the entire microfluidic channel is exposed to a solution of enzyme during this process, nonspecific adsorption of enzyme outside of the desired immobilization region can prevent effective patterning of the enzyme. These groups limited nonspecific adsorption of enzyme through the use of blocking protein. Another limitation of this technique is that the amount of immobilized enzyme is limited by the available surface area of the channel. Several groups have attempted to increase the amount of immobilized enzyme by entrapping enzyme within photopatterned hydrogels.[30−33] By this method, enzyme is encapsulated within a gel rather than attached covalently. The gels must be dense enough to retain protein, thus rendering the enzyme inaccessible to convective flow. Consequently, although more enzyme is immobilized, not all of the immobilized enzyme is necessarily available for reaction. Furthermore, the use of dense gels can significantly increase the flow resistance in a microchannel.

The present study develops a method of photopatterning multiple enzymes in microfluidic devices at high densities in order to enable multistep enzymatic reactions whose reaction sequence is determined by the direction of fluid flow. The immobilization method employed in this work was the covalent attachment of enzyme to the surface of a porous polymer monolith. Porous polymer monoliths have been developed over the past decade as support materials for a variety of analytical applications.[34] Polymer monoliths increase the available surface area in a microchannel and are thus particularly useful for high-density enzyme immobilization.[6,35−39] In addition, porous polymers are easy to fabricate in-device via a UV-initiated polymerization process whereby liquid monomers polymerize in the presence of porogenic solvents to form a porous solid support that can be patterned using a photomask.[40,41] Furthermore, because they are covalently anchored to the microchannel walls, polymer monoliths do not require any retaining structures such as the microfabricated weirs that are needed to retain microbeads. Recently, we introduced a photografting method for the in situ modification of these porous polymers.[42,43] This surface modification process is also photoinitiated, allowing the polymer surface to be photopatterned with specific chemical functionalities such as azlactone for the covalent attachment of a variety of proteins.[38,42] In particular, we have used this technique to fabricate a simple monolithic device with dual functionality that combined solid-phase extraction and tryptic digestion.[38] However, this early approach enabled only limited control over the nonspecific adsorption of enzyme throughout the device. Recently, we extended our photografting technique to poly(ethylene glycol) methacrylates in order to obtain protein adsorption-resistant coatings.[44]

In this work, we demonstrate the preparation of monolithic porous polymer supports that suppress the nonspecific adsorption of enzymes and enable the photopatterning of spatially separated multienzymatic microreactors. Kinetic parameters for an immobilized enzyme reaction are determined by measuring product formation on device and analyzing the results using a plug-flow reactor model. Finally, we demonstrate model sequential reactions involving two and three enzymes, and we present an example of directional biosynthesis.

EXPERIMENTAL SECTION

Materials. Butyl methacrylate (99%, BuMA), ethylene dimethacrylate (98%, EDMA), 1-dodecanol (98%), cyclohexanol (99%),

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benzophenone (99%, BP), 3-(trimethoxysilyl)propyl methacrylate, ethanolamine (98%), and 2,2-dimethoxy-2-phenylacetophenone (99%) were purchased from Sigma-Aldrich Co. (St. Louis, MO). BuMA and EDMA were purified by distillation under reduced pressure to remove inhibitors. Ethoxylated hydroxyethyl methacrylate (PEGMA, average M_n = 570 g/mol, containing 10−11 ethylene glycol units) was purchased from Sartomer (Exton, PA). PEGMA was purified by passing it through a column containing inhibitor remover beads of basic alumina (Aldrich). 2-Vinyl-4,4-dimethylazlactone (vinyl azlactone) was a gift from the 3M Co. (St. Paul, MN). UV-transparent fused-silica capillary (100-μm i.d.) was purchased from Polymicro Technologies (Phoenix, AZ). Fused-silica capillaries served as model microfluidic channels in this study.

Glucose oxidase (EC 1.1.3.4, ~200 units/mg from *Aspergillus niger*), horseradish peroxidase (EC 1.11.1.7, 250−330 units/mg from horseradish), and invertase (INV, EC 3.2.1.26, >300 units/mg from *Saccharomyces cerevisiae*) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Recombinant green fluorescent protein (GFP) was produced by the method described by Whitehead et al.[45] Sodium phosphate monobasic, sodium chloride, and sodium hydroxide were purchased from Fisher Scientific (Hampton, NH). Resorufin dye, 3 wt % hydrogen peroxide, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. The concentration of hydrogen peroxide was regularly assessed with a hydrogen peroxide kit from Palintest (Erlanger, KY). Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine) was purchased from Invitrogen Corp. (Carlsbad, CA). A stock solution of 10 mmol/L Amplex red was prepared by dissolving 5 mg of Amplex Red in anhydrous DMSO; the resultant solution was stored protected from light at −20 °C.

Instrumentation. An OAI model 30 deep UV collimated light source (San Jose, CA) fitted with a 500-W HgXe lamp was used for UV exposures. The irradiation power was calibrated to 15.0 mW/cm^2 using an OAI model 306 UV power meter with a 260-nm probe head. Microscopic evaluations were performed using a Nikon TE200 inverted fluorescence microscope (Scientific Instrument Co., Sunnyvale, CA). Images were acquired with a Micropublisher 5.0 RTV CCD camera from QImaging (Burnaby, BC, Canada). Green fluorescence was observed with Nikon filter B-1A; red fluorescence was observed with Nikon filter TRITC HYQ. Image analysis and quantification was performed with image analysis program ImageJ, provided by the NIH. Parameter fitting was performed by linear multiple regression performed using scientific graphing and data analysis software, Origin (OriginLab Corp., Northampton, MA). Off-chip fluorescent assays were analyzed with a SpectraMax M2 fluorescent microplate reader (Molecular Devices Corp., Sunnyvale CA).

Capillary Surface Activation. The inner surface of fused-silica capillaries was first activated with 3-(trimethoxysilyl)propyl methacrylate; this process enables the polymer monolith to covalently attach to the capillary surface through the resulting pendent vinyl groups. The inner capillary surface was first quickly rinsed with acetone and water, then flushed with 0.2 mol/L sodium hydroxide for 30 min at a flow rate of 0.5 μL/min, and quickly rinsed with water. Next, the capillary was flushed with 0.2 mol/L hydrochloric acid for 30 min at a flow rate of 0.5 μL/min, rinsed with water, and finally flushed with ethanol. A 20 wt % solution of 3-(trimethoxysilyl)propyl methacrylate in 95% ethanol with pH adjusted to 5 using acetic acid was pumped through the capillary at a flow rate of 0.5 μL/min for 60 min. The capillary were then washed with acetone, dried in a stream of nitrogen, and left at room temperature for 24 h.

Monolith Formation. The polymerization mixture contained the monomers BuMA and EDMA and porogens 1-decanol and cyclohexanol. The polymerization mixture consisted of 24 wt % BuMA, 16 wt % EDMA, 50 wt % 1-decanol, 10 wt % cyclohexanol, and 1 wt % DMPA (with respect to monomers) and was purged with nitrogen for 10 min prior to use. A 10-cm-long capillary that had been previously activated was filled with this mixture, placed under the light source, and irradiated with UV light for 10 min at a distance of 30 cm from the UV source. After photopolymerization, the porogenic solvents were flushed from the polymer monolith by pumping methanol through the column at a flow rate of 0.5 μL/min for 8 h. The average pore size of the monolith was ~1.6 μm, as measured by mercury intrusion porosimetry.[42]

Photografting of PEG. Poly(ethylene glycol) (PEG) was photografted onto the surface of the methacrylate polymer monolith to render it hydrophilic and prevent the nonspecific adsorption of protein to the monolith surface.[44] Briefly, BP was initially grafted to the surface of the monolith by rinsing the monolith with a 5 wt % BP solution in methanol at a flow rate of 0.5 μL/min for 30 min and then exposing the monolith to UV irradiation for 2 min. After photografting BP, the monolith was rinsed with methanol at a flow rate of 0.5 μL/min for 20 min to remove unbound initiator. Next, a 0.10 mol/L solution of PEGMA monomer in water was pumped at a flow rate of 0.5 μL/min through the monolith for 30 min. The monolith was then exposed to UV irradiation for 2 min. The monolith was then rinsed with water at a flow rate of 0.5 μL/min for 7 h to remove any unreacted hydrophilic monomers.

Photografting of Vinyl Azlactone. To enable the covalent immobilization of proteins, the surface of the polymer monolith was first activated by photografting with the reactive monomer 2-vinyl-4,4-dimethylazlactone, using a procedure modified from previous reports.[36,44] The photografting solution consisted of 15 wt % vinyl azlactone and 0.22 wt % BP dissolved in a solution of 75:25 wt % *tert*-butyl alcohol/water. Vinyl azlactone was photografted onto the surface of the monoliths using a 5-min exposure through a photomask; the mask defined the area where protein was to be immobilized. After photografting vinyl azlactone, the monolith was rinsed with acetone for 1 h at a flow rate of 0.5 μL/min to remove excess reagents.

Protein Immobilization. Protein was covalently immobilized onto the surface of a monolith already photografted with vinyl azlactone by pumping a protein solution through the monolith at a flow rate of 0.5 μL/min for 1−1.5 h. The azlactone functionality reacts with the amine groups of proteins, primarily the ϵ-amino group of lysine, forming a covalent bond between the protein and the support surface. The monolith was then rinsed with 1.0 mol/L ethanolamine at 0.5 μL/min for 1 h to quench unreacted azlactone functionalities. Finally, the capillary was rinsed with 50 mmol/L phosphate buffer, pH 7.50, at 1.0 μL/min for 1 h to remove excess reagents.

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Two-Enzyme Assay. HRP was initially patterned on 1 cm of a 9-cm-long monolith via the previously described vinyl azlactone photografting and enzyme immobilization process. GOX was then patterned on the other 8 cm of the monolith, by performing a second round of patterned vinyl azlactone photografting, followed by GOX immobilization. The GOX immobilization solution consisted of 10 mg/mL GOX and 0.01% (v/v) Tween-20 in 50 mmol/L phosphate buffer, pH 7.50. After each enzyme was immobilized, the monolith was rinsed with 1.0 mol/L ethanolamine at 0.5 μL/min for 1 h to quench any unreacted azlactone functionalities. Finally, the monolith was rinsed with phosphate buffer at 1.0 μL/min for 1 h. A 1.0 mg/mL dextrose solution was created by dissolving crystalline dextrose in phosphate buffer. This solution was allowed a minimum of 12 h to establish equilibrium between the isomers of glucose. Immediately prior to use, the dextrose solution was saturated with pure oxygen for a minimum of 15 min. To 1.00 mL of this dextrose solution, 10 μL of the 10 mmol/L stock Amplex Red solution was added such that the final concentration was 100 μmol/L Amplex Red and 1.0% (v/v) DMSO. A 50-μL sample was collected and the fluorescence was analyzed as previously described. Between trials the monolith was rinsed with 50 μL of phosphate buffer at 1.0 μL/min to remove excess reagents.

Three-Enzyme Assay. Similar to the two-enzyme procedure, a 9-cm-long monolith was initially patterned with 1 cm of HRP. GOX was then patterned on 4 cm, and finally INV was patterned to the remaining 4 cm of the column, using a third round of photopatterned vinyl azlactone grafting, followed by INV immobilization. The INV immobilization solution consisted of 10 mg/mL INV and 0.01% (v/v) Tween-20 in 50 mmol/L phosphate buffer, pH 7.50. After each enzyme was immobilized, the monolith was rinsed with 1.0 mol/L ethanolamine at 0.5 μL/min for 1 h to quench unreacted azlactone functionalities. Finally, the monolith was rinsed with phosphate buffer at 1.0 μL/min for 1 h. A 10.0 mg/mL sucrose solution was prepared by dissolving crystalline sucrose in phosphate buffer. Immediately prior to use, the sucrose solution was saturated with pure oxygen for a minimum of 15 min. To 1.00 mL of this sucrose solution, 10 μL of the 10 mmol/L stock Amplex Red solution was added such that the final concentration was 100 μmol/L Amplex Red and 1.0% (v/v) DMSO. A 50-μL sample was collected at a flow rate of 0.10 μL/min, and the fluorescence was analyzed as previously described. Between trials, the monolith was rinsed with 50 μL of phosphate buffer at 1.0 μL/min to remove excess reagents.

RESULTS AND DISCUSSION

Minimizing Nonspecific Protein Adsorption. Successful patterning of immobilized enzymes requires the suppression of nonspecific protein adsorption, which can result in the immobilization of active enzyme outside of a patterned region. Minimizing nonspecific protein adsorption is particularly important with high-surface-area support materials such as porous polymer monoliths. To this end, horseradish peroxidase was employed to investigate the nonspecific adsorption of protein onto the surface of the polymer monoliths and to evaluate the effectiveness of attempts to pattern enzyme immobilization. The activity of HRP was detected using the fluorogenic substrate, Amplex Red. HRP catalyzes the oxidation of nonfluorescent Amplex Red to fluorescent resorufin, employing hydrogen peroxide as a cosubstrate in

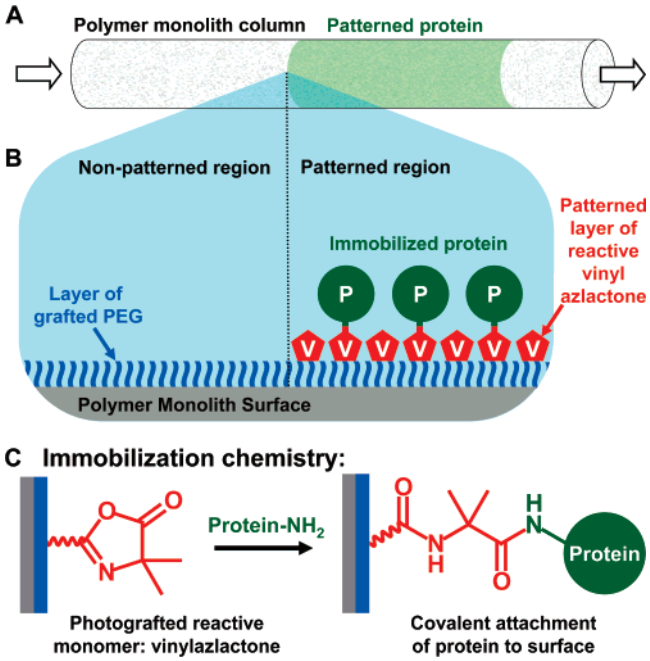

Figure 1. Schematic diagram of the photopatterning process. (A) Protein is immobilized to the surface of a polymer monolith in patterned regions within a microfluidic channel. (B) PEG is grafted to the surface of the polymer monolith to prevent nonspecific protein adsorption. Vinyl azlactone is photopatterned onto the PEG surface and activates the surface for protein immobilization. (C) Azlactone functionality reacts with amines of proteins to form a covalent amide bond between the protein and the polymer monolith surface.

a 1:1 stoichiometry.[46] Monitoring any fluorescence produced within the microchannels provides a sensitive means of detecting the presence of active enzyme.

We employed azlactone attachment chemistry for covalent immobilization of enzyme to the polymer monolith supports, whereby reactive azlactone groups are introduced onto the monolith surface by photografting the surface with vinyl azlactone.[38,42] The resulting azlactone functionalities react with amines on proteins, as illustrated in Figure 1; the coupling reaction is relatively rapid and not overly sensitive to hydrolysis.[48] No leaving groups are produced during the immobilization reaction since azlactone reacts via a ring-opening, nucleophilic addition. In addition, the azlactone attachment chemistry does not require any modifications of the enzyme prior to immobilization such as those required with the frequently used streptavidin−biotin bioconjugation techniques.[25,28] An important benefit of photografting is that it is possible to pattern the location of grafted azlactone and, subsequently, the location of immobilized protein (Figure 1). Furthermore, multiple enzymes can be immobilized in different locations within a single device by repeating the azlactone photografting and subsequent enzyme immobilization process for each enzyme.

In order to determine appropriate immobilization conditions, pairs of identical polymer monolith columns were prepared in

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Table 1. Effect of Surface Treatment on the Concentration of Resorufin Produced from Immobilized HRP on Patterned and Nonpatterned Monolithic Columns[a]

monolith type	patterned monolith [resorufin] (μmol/L)	nonpatterned monolith [resorufin] (μmol/L)	ratio of [resorufin] patterned/nonpatterned
initial monolith	7.8 ± 1.3	7.6 ± 1.5	1.0
PEG-grafted monolith	6.4 ± 1.1	0.88 ± 0.02	7.3

[a] The reported error is the standard deviation of three separate trials. Conditions: patterned and nonpatterned monolith columns, 4.5 cm in length; HRP was immobilized to 1.0 cm of the patterned monolith; enzyme immobilization solution, 1.0 mg/mL HRP in 50 mmol/L phosphate buffer, pH 7.50; substrate solution, 8.80 μmol/L hydrogen peroxide, 100 μmol/L Amplex Red, and 0.10% (v/v) DMSO in 50 mmol/L phosphate buffer, pH 7.50; substrate flow rate, 1.0 μL/min; 50 μL of sample was collected and analyzed.

which one column contained a photopatterned region of grafted vinyl azlactone for enzyme immobilization, as in Figure 1A and B, while the other column was not patterned with azlactone. Then, both columns were treated with the enzyme immobilization solution. Thus, enzyme was covalently immobilized to the photopatterned region of one column, whereas the only enzyme present in the other column was the result of nonspecific adsorption. With unmodified polymer monolith supports, both the patterned and nonpatterned columns exhibited a high level of enzyme activity (Table 1), indicating that nonspecific adsorption onto the polymer monolith was significant and that much of the adsorbed enzyme remained active.

Several attempts were made to disrupt the surface interactions that resulted in the high degree of nonspecific HRP adsorption. Following enzyme immobilization, the monoliths were rinsed with a high ionic strength solution (1.0 mol/L NaCl) to disrupt ionic interactions possibly responsible for nonspecifically adsorbed protein and rinsed with solutions of pH 6.0 and pH 9.0 to vary the net charge of the HRP. Neither of these steps significantly decreased the observed activity of the nonpatterned monoliths, indicating that nonspecific adsorption of HRP was not due primarily to ionic interactions.

Next, to examine the impact of nonionic interactions, specifically hydrophobic interactions, PEG was grafted to the surface of the monolith in the first step followed by photografting vinyl azlactone on the top of the PEG layer. We have demonstrated the utility of this two-layer approach previously.[43] PEG grafting imparts a hydrophilic character to the surface of the monolith and has previously been shown to reduce the nonspecific adsorption of protein on polymer monoliths.[44] The PEG surface treatment reduced the amount of resorufin produced from both columns, but a much larger reduction was observed for the nonpatterned column. The benefit of PEG grafting on reducing protein adsorption is clearly reflected in the ratios of the amount of resorufin produced from the patterned columns over that produced by the nonpatterned columns. The value for unmodified monoliths was 1.0, while the PEG-grafted columns exhibited a ratio of 7.3.

Next, we investigated the effect of enzyme concentration, which influences both the immobilization of enzyme and the extent of nonspecific adsorption. To evaluate this effect, patterned and nonpatterned PEG-grafted monolithic columns were prepared using immobilization solutions with different concentrations of HRP. The greater the concentration of HRP in the immobilization solution, the larger the amount of resorufin produced for both the patterned and nonpatterned monolith columns (Table 2). Maximizing the activity of the patterned column through the use of a higher HRP concentration must be balanced against minimizing nonspecific protein adsorption, which is facilitated by employing a lower HRP concentration in the immobilization solution. For the conditions investigated, the HRP concentration of 0.10 mg/mL best balanced these divergent trends and maximized the ratio of patterned/nonpatterned product formation.

Table 2. Effect of HRP Concentration in Immobilization Solution[a]

[HRP] (mg/mL)	patterned monolith [resorufin] (μmol/L)	nonpatterned monolith [resorufin] (μmol/L)	ratio of [resorufin] patterned/ nonpatterned
1.0	6.4 ± 1.1	0.88 ± 0.02	7.3
0.10	3.6 ± 0.62	0.32 ± 0.06	11
0.010	0.54 ± 0.04	0.28 ± 0.06	1.9

[a] For conditions, see Table 1.

Table 3. Effect of Surfactant Concentration in Immobilization Solution[a]

[Tween-20] (%) (v/v)	patterned monolith [resorufin] (μmol/L)	nonpatterned monolith [resorufin] (μmol/L)	ratio of [resorufin] patterned/ nonpatterned
0.10	0.62 ± 0.02	0.50 ± 0.02	1.2
0.010	5.6 ± 0.12	0.32 ± 0.04	18

[a] The concentration of HRP in the immobilization solution was 0.10 mg/mL. For other conditions, see Table 1.

Finally, Tween-20, a common surfactant, was included in the immobilization solution to further disrupt hydrophobic interactions and reduce the nonspecific adsorption of HRP on the PEG-grafted polymer monoliths. The activity of the patterned column remained high when the Tween-20 concentration was 0.010% (v/v). However, at 0.10% (v/v), the activity of the patterned column was significantly reduced (Table 3). The use of a high concentration of Tween-20 likely had an adverse effect on the activity of the immobilized HRP.[49] However, PEG grafting in combination with the appropriate HRP and Tween-20 concentrations (0.10 mg/mL and 0.010% (v/v), respectively) resulted in a patterned/nonpatterned column ratio of 18. Thus, these techniques for reducing nonspecific protein adsorption enabled the effective patterning of protein to spatially defined regions within a microfluidic channel.

In Situ Measurement of Enzyme Kinetics Using Continuous Flow. In order to measure in situ steady-state fluorescent product profiles, HRP and GFP were coimmobilized to a small, patterned region on the surface of a PEG-grafted monolith. GFP

(49) Kenny, G. E.; Dunsmoor, C. L. *Isr. J. Med. Sci.* **1987**, *23*, 732−4.

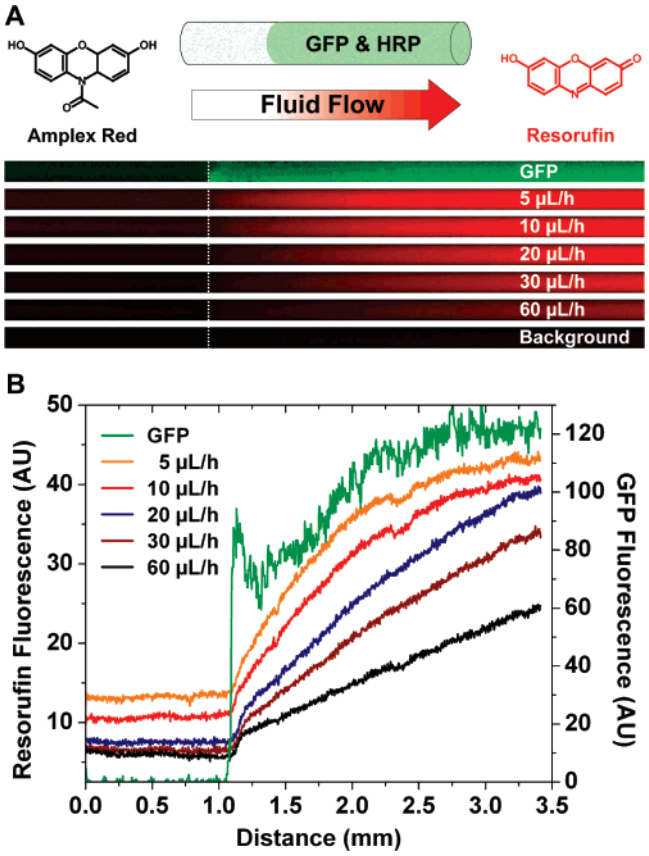

Figure 2. (A) Steady-state formation of fluorescent resorufin from Amplex Red catalyzed by photopatterned HRP immobilized on polymer monolith in a 100-μm capillary (substrate solution flow is from left to right). The top fluorescent image shows the green fluorescence of GFP, which coincides with the location of the coimmobilized patterned HRP. The other fluorescent micrographs show the red fluorescence of resorufin produced in the microreactor. Conditions: substrate solution, 8.80 μmol/L hydrogen peroxide, 100 μmol/L Amplex Red, and 1.0% (v/v) DMSO in 50 mmol/L phosphate buffer, pH 7.50. (B) Steady-state resorufin fluorescence intensity profiles taken from the images in (A). The fluorescence intensity at each point in the microreactor column was determined by averaging the fluorescence intensity across the cross section of the monolith. The fluorescence profile of GFP indicates the location of the coimmobilized HRP.

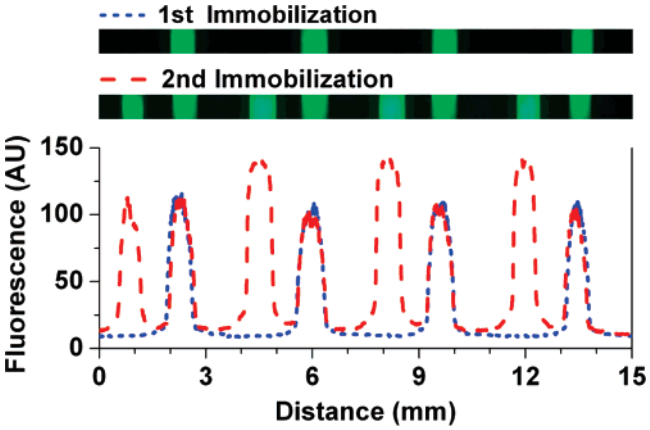

Figure 3. Consecutive patterning of multiple GFP patches on a polymer monolith in a 100-μm capillary. The top image shows the location of GFP simultaneously immobilized in multiple patches; each GFP patch is ~750 μm in length. The lower image shows the fluorescence from GFP patches after a second round of GFP immobilization on the same monolith. The plot shows profiles of the GFP fluorescence along the length of the capillary for the above images; the fluorescence intensity at each point in the column was determined by averaging the fluorescence intensity across the cross section of the monolith. In the images above, the diameter of the monolith has been increased over the actual aspect ratio to more clearly show the patch positions.

was included to enable direct visualization of the immobilized protein. Control experiments determined that including 0.10 mg/mL GFP did not measurably affect the activity of the immobilized HRP. Fluorescent images of the patterned GFP and the steady-state production of resorufin at flow rates ranging from 5.0 to 60 μL/h are shown in Figure 2. A significant increase in resorufin fluorescence coincided with the location of the patterned protein, as evidenced by the GFP fluorescence, confirming that active HRP was photopatterned with little nonspecific adsorption. Two additional observations were that (i) for a given flow rate the resorufin fluorescence increased along the length of the microreactor and (ii) lower flow rates produced a higher level of resorufin fluorescence. Both of these observations can be explained by the effect of the residence time of the substrate, Amplex Red, in the reactor. For a given flow rate, the residence time increases with increasing distance along the length of the microreactor; while for a given position in the microreactor, residence time increases as the flow rate decreases. One final observation is that the resorufin fluorescence prior to the microreactor increases with decreasing flow rates. This phenomenon can be attributed to the activity of remaining nonspecifically adsorbed HRP upstream of the microreactor. The steady-state product profiles were used to determine the relationship between reaction conversion and residence time for the immobilized HRP. Detailed description of the measurements and their results are presented in Supporting Information.

Directional Synthesis in Spatially Separated Multienzymatic Reactions. Photopatterning allows multiple proteins to be independently positioned within a single microfluidic channel. Such a configuration enables multistep sequential enzymatic reactions to occur within well-defined spatial reaction zones with a reaction sequence that is determined by the direction of fluid flow. We demonstrate the ability to pattern protein by consecutively photopatterning multiple patches of GFP, with one set of patches slightly offset from the other, as shown in Figure 3. The fluorescent intensity of the patterned GFP revealed that the second round of photopatterning did not denature the initial GFP patches, nor did it result in additional GFP binding to the original patches.

Patterning enzymes in microfluidic systems also enables control of the sequence of multienzymatic reactions. We demonstrate directional synthesis using a model two-enzyme reaction involving GOX and HRP. In the first step of the reaction, GOX employs dissolved oxygen to catalyze the oxidation of β-D-glucose to δ-D-glucono-1,5-lactone and hydrogen peroxide. The hydrogen peroxide is then utilized by HRP in the second step of the reaction to oxidize Amplex Red to resorufin. The enzymes were patterned by first immobilizing HRP to one end of a monolith, followed by immobilization of GOX to the other end. The patterned GOX section was 8 times longer than the patterned HRP section to achieve a longer residence time with GOX and compensate for the relatively slow GOX reaction rate observed in separate comparisons of the two enzymes (data not shown). A substrate

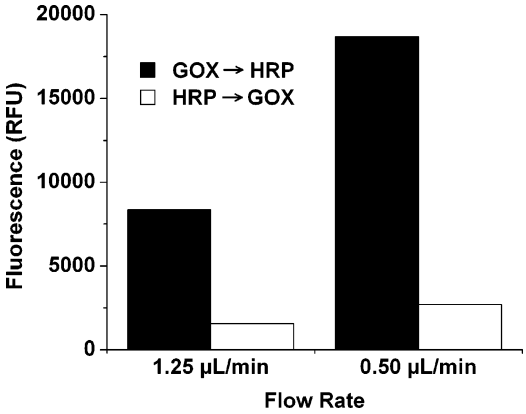

Figure 4. Directional synthesis with a two-enzyme system: GOX and HRP. Conditions: monolithic support, 9 cm long; GOX and HRP patterned to a length of 8 and 1 cm, respectively; product fluorescence measured in both the forward (GOX → HRP) and reverse (HRP → GOX) directions; substrate solution, 1.0 mg/mL dextrose, 100 μmol/L Amplex Red, and 1.0% (v/v) DMSO in 50 mmol/L phosphate buffer, pH 7.50; pure oxygen was bubbled through this solution for 15 min prior to use.

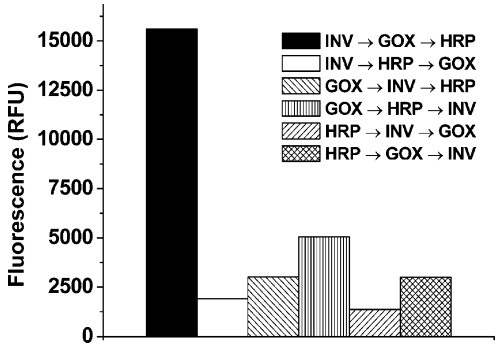

Figure 5. Directional synthesis with a three-enzyme system: INV, GOX, and HRP. Conditions: monolithic support, 9 cm long; INV, GOX, and HRP patterned to a length of 4, 4, and 1 cm, respectively; Product fluorescence was measured from columns prepared with each possible arrangement of the three enzymes, as indicated in the figure legend; The substrate solution consisted of 10 mg/mL sucrose, 100 μmol/L Amplex Red, and 1.0% (v/v) DMSO in 50 mmol/L phosphate buffer, pH 7.50; pure oxygen was bubbled through this solution for 15 min prior to use. The flow rate was 0.10 μL/min.

solution consisting of glucose and Amplex Red was flushed through the capillary in forward and reverse directions, and the results of these experiments are plotted in Figure 4. As previously observed, the concentration of fluorescent product increased with residence time. More important, the direction of fluid flow substantially affected the fluorescence. When the substrate solution passed through the column in the forward direction, i.e., from GOX to HRP, the fluorescence was much higher than when then same solution flowed in the reverse direction. The minimal fluorescence observed in the reverse flow direction was likely due to the attachment of either GOX or HRP outside of their defined patterned regions. This attachment may be attributed to effects such as nonspecific adsorption to the monolith, noncovalent interactions with previously immobilized enzyme, and covalent attachment to residual azlactone groups.

A sequential three-enzyme reaction was also performed. In the first step, INV hydrolyzes sucrose to glucose and fructose; in the second step, GOX oxidizes glucose to gluconolactone and hydrogen peroxide, which is then used by HRP to oxidize Amplex Red in the third step. Monolithic microreactors were created such that the INV and GOX sections were each 4 times longer than the HRP section. Several microreactors were prepared with all six possible arrangements of the three enzymes. A substrate solution consisting of sucrose and Amplex Red was then flushed through the monoliths and the samples were analyzed; the results are presented in Figure 5. The correct sequential order of catalyst (INV− GOX−HRP) produced the largest resorufin fluorescence by more than 3-fold. Although these studies were carried out across several weeks, no significant change in the enzymatic activity was observed.

In this case, a third enzyme was easily added to the same microchannel. In the same way, additional enzymes could be photopatterned within a single microchannel to create a multienzyme system. By comparison, including an additional enzyme in a spatially separated system based on microbeads would require redesign of the entire microfluidic device.

SUMMARY AND CONCLUSIONS

We have developed a simple strategy to immobilize several enzymes in different and well-defined regions of a microfluidic device by a photopatterning method involving a high-surface-area polymer monolith support. Photoinitiated grafting of selected monomers enabled activation of the support for immobilization and reduction of nonspecific protein adsorption. Use of the in situ prepared monolithic supports provides the increased surface area without the need for separate retaining structures required when using microbeads. The steady-state product profiles of a patterned enzyme were measured and utilized to determine enzyme kinetics in situ. It is anticipated that similar measurements could be performed in a high-throughput format to determine the kinetic properties for large enzyme libraries. The same patterning technique was also employed to demonstrate directional biosynthesis with model two- and three-enzyme reactions. Potential applications of photopatterned enzyme sequences include miniaturized analytical and diagnostic assays utilizing a specific order of reactions, high-throughput combinatorial biocatalysis in a microfluidic format, and the creation and investigation of artificial metabolic pathways *ex situ*.

ACKNOWLEDGMENT

Financial support of this work by the National Institute of Biomedical Imaging and Bioengineering, National Institutes of Health (EB-006133), is acknowledged with thanks. T.C.L. and D.S.C. recognize partial support of this work by the UC Discovery Grant, the Industry—University Cooperative Research Program, and Genencor International. Work at the Molecular Foundry was supported by the Director, Office of Science, Office of Basic Energy Sciences, Materials Sciences and Engineering Division, of the U.S. Department of Energy under Contract DE-AC02-05CH11231. T.C.L. and T.B.S. contributed equally to this work.

SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review April 10, 2007. Accepted June 20, 2007.

AC070705K